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Investigation of How Hydrogen Bonding Affects the Enantiomeric Excess of Pig Liver Esterase Promoted Hydrolysis of Pro-Chiral Substrates

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The University of Southern Mississippi

Investigation of How Hydrogen Bonding Affects the Enantiomeric Excess of Pig Liver
Esterase Promoted Hydrolysis of Pro-Chiral Substrates

by

Jacob Pruett

A Thesis
Submitted to the Honors College of
The University of Southern Mississippi
in Partial Fulfillment
of the Requirements for the Degree of
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in the Department of Chemistry and Biochemistry

May 2016

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Abstract

Pig Liver Esterase is a cost effective enzyme for ester hydrolysis. In our group, it is vital for creating chiral molecules for the synthesis of unnatural amino acids of potential biological importance. It has been previously found that the enantiomeric excess (%ee) of the PLE hydrolysis reaction increases drastically with the addition of co-solvents that are able to both accept and donate hydrogen bonds. This research endeavors to see if substrates of enhanced hydrogen bonding ability also increase the stereoselectivity of PLE hydrolyses. Diester malonate was covalently linked with a furan ring in both the third and second position from the oxygen atom to test this. These two substrates are unable to donate hydrogen bonds, but they are able to accept them. It was found that the substrate with the furan in the second position gave an %ee of 70% with no added co-solvent while the substrate in the third position gave a racemic mixture with no added co-solvent. This hints that there may be an amino acid anchoring the substrate in the active site of PLE, which will favor the creation of one enantiomer over the other. When 2.0% ethanol co-solvent was used in the PLE hydrolysis reaction the %ee rose to around 35%. To complete the series, diester malonate will be combined with a pyrrole ring in the second position from the nitrogen atom, which can only donate hydrogen bonds. This substrate will then undergo PLE hydrolysis with and without co-solvent to see the reactions' respective enantiomeric excesses.

Keywords: PLE, Stereoselectivity, Hydrogen Bonding

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List of Abbreviations

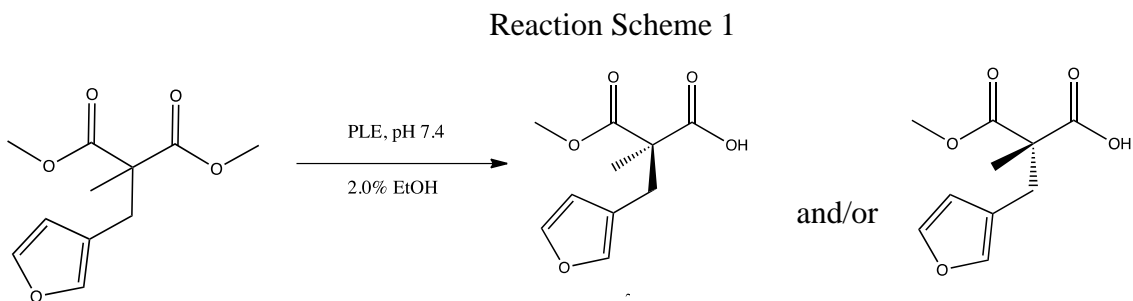
PLE	Pig Liver Esterase
HL	Long Hydrophobic
Hs	Short Hydrophobic
P_B	Back Polar
P_F	Front Polar
%ee	Enantiomeric Excess
¹H-NMR	Proton Nuclear Magnetic Resonance
¹³C-NMR	Carbon-13 Nuclear Magnetic Resonance
IR	Infrared
HRMS	High-Resolution Mass Spectrometry
HPLC	High-Performance Liquid Chromatography
TLC	Thin Layer Chromatography
NaOH	Sodium Hydroxide
HCl	Hydrochloric Acid
NaH	Sodium Hydride
NaBH₄	Sodium Borohydride
THF	Tetrahydrofuran
CH₃I	Iodomethane
EtOH	Ethanol

Introduction

A chiral center is a carbon that is attached to four different substituents. The formation of a chiral center in an organic synthesis is often a cause for internal groaning for organic chemists. When a compound has one chiral center, there are two possible enantiomers. One enantiomer will rotate plane-polarized light in a clockwise direction while the other will rotate it in a counterclockwise direction. This was first noted by Louis Pasteur regarding (+)-tartaric acid and (-)-tartaric acid, which he managed to separate from one another in 1848 via crystallization (Gal, 2008). In a flask, nature tends to make both enantiomers equally. However, many compounds, such as amino acids and drugs, require specific chiral centers. In order to avoid wasting approximately 50% of the product, chemists are looking for methods to favor one enantiomer over the other. One of those methods this study researched was a bioorganic synthesis. Most of the reactions for our synthesis were exclusively organic chemistry, but the formation of the chiral center was bioorganic because of the use of an enzyme. Enzymes have been found to be specific in what they make, especially when forming enantiomers. Therefore, we used Pig Liver Esterase (PLE) kept at a pH of 7.4 for the formation of our chiral center. Our central hypothesis is that hydrogen bonding between the substrate and PLE aids in the production of one enantiomer over the other.

The goal of our research is to find a way to investigate whether or not hydrogen bonding in a substrate can improve the enantiomeric excess of the product of a PLE hydrolysis. Biological reactions regularly require a specific spatial configuration of their compounds of interest, their substrates. While PLE was not *in vivo*, it reacted with our substrate *in vitro* to make a product with a chiral carbon. When a carbon has four

different groups attached to it, it becomes a chiral carbon. When one chiral carbon is present in a compound, a pair of enantiomers can exist.



When a chiral center is created, then it is imperative to measure the enantiomeric excess of the product mixture to determine how enantiomerically pure the product is. Enantiomeric excess is a percentage of the excess of one enantiomer made compared to the other enantiomer made. Enantiomeric excess is equal to the difference between the moles of each enantiomer made divided by the total moles of both enantiomers made, all multiplied by 100. The higher the enantiomeric excess, the more enantiomerically pure the product is and the more stereoselective the reaction is perceived to be (Fryhle and Solomons, 2011).

Pig Liver Esterase, while at a pH of 7.4 with phosphate buffer, has been found to make largely one enantiomer over the other possible enantiomer with diester substrates (substrates with two ester functional groups) that have an α quaternary carbon. In our reactions, crude PLE is used, which is a ground up pig liver extract which contains all six isoenzymes of PLE. It has been found that PLE works significantly better with a co-solvent. It has been noted that these co-solvents can hydrogen bond, and it would be interesting to see if the enantiomeric excess of the product from PLE hydrolysis would increase if the diester substrate had a moiety that could participate in a hydrogen bond.

We made compounds that were diesters with a furan moiety which could participate in hydrogen bonding. Those diesters then underwent hydrolysis with hydroxide in one condition as a control group (Reaction Scheme 7), and then underwent hydrolysis with crude PLE (Reaction Scheme 1) in order to compare the enantiomeric excess and stereochemistry of each resulting product.

The product of the PLE hydrolysis could be carried on and made into an unnatural amino acid by undergoing first a Curtius rearrangement and then a NaOH hydrolysis (Smith et al, 2012). Having a furan ring as a part of the R group of the amino acid would make it an interesting tool in engineering proteins as this amino acid would be both hydrophobic and capable of hydrogen bonding. Also, the furan ring could potentially undergo a Diels-Alder reaction that could give a protein a different overall shape by making its ends join together, or it could even join two proteins together. It should be noted that actually testing the furan moiety's ability to help engineer proteins would be beyond the scope of this project.

Literature Review

Importance for Specific Enantiomers

It is often said that structure equals function. For instance, it has been found that cyclizing some linear peptides aided in their antibacterial function (Anderson et al, 2010). Much of the metabolism in our body, the way the human body builds up or breaks down chemicals, is mediated by enzymes. These enzymes tend to be stereospecific because they are composed of amino acids, most of which are chiral. This was first hinted at when Pasteur discovered that tartrate fermentation in *Penicillium glaucum* was enantioselective (Gal, 2008). Therefore, when constructing a new drug or some other biologically relevant

compound, the stereochemistry of the compound is often nonnegotiable. One enantiomer could lead to the desired biological effect while the other could lead to undesired consequences. A famous example is of the thalidomide incident, where one enantiomer cured morning sickness while the other enantiomer caused devastating birth defects (Guo, 2011). While the consequences of marketing a racemic product to the public may not always be so drastic, the thalidomide incident showed that a compound's stereochemistry cannot be discounted.

This study originally focused on trying to make one enantiomer over the enantiomer so that biologically relevant amino acids could be created. However, as the research progressed, this became a secondary goal. The primary goal of this research is to make probes to test PLE to see how it responds to probes that contain heteroatoms of varying hydrogen bonding ability in different positions on the probe.

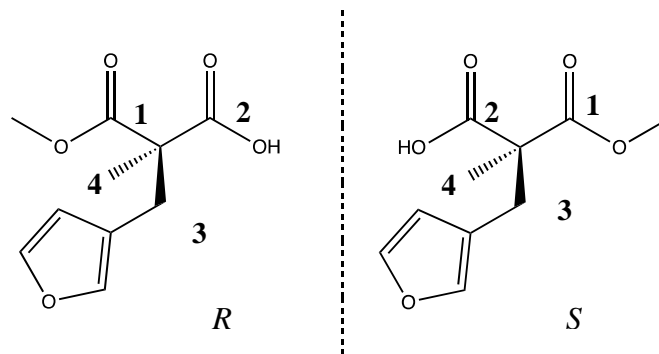


Figure 1: Enantiomers

The above image shows the compound on the left (the *R* enantiomer) inverted to look like the compound on the right (the *S* enantiomer). The dashed line represents a mirror, so that if the *R* enantiomer were reflected in a mirror, one would see the *S* enantiomer. However, the *R* and the *S* enantiomers are non-superimposable. A simple way to see the difference is if one envisioned a pair of shoes. If one took a pair of shoes,

they would find that they could not be stacked on top of each other and match exactly. In the same way, enantiomers cannot be superimposed on each other either.

In order to determine if an enantiomer is *R* or *S*, the four groups around the carbon must be assigned priorities. Once the group of least priority has been placed going away from the plane, the *R* enantiomer is shown having the other groups organized from highest priority to third-lowest priority in a clockwise rotation. The *S* enantiomer has a counterclockwise rotation of those groups (Fryhle and Solomons, 2008). Enantiomers have the same physical properties as each other, such as melting point and boiling point, but the way they fit into enzymes and optically rotate differ (Gal, 2008).

Not every compound has a pair of enantiomers. In order to have a pair of enantiomers, a compound needs at least one chiral center. A chiral center is a carbon connected to four different groups (Gal, 2008). There are some other cases for chiral centers, but those are beyond the scope of this research.

Making Specific Enantiomers

Since chiral centers are often needed to make medications to give to patients, a synthesis is needed in order to assure that only one enantiomer is created. Otherwise, money is wasted in losing about half of the product. (Without chiral catalysts like enzymes, nature likes to create both enantiomers in equal amounts upon the creation of a chiral center). Furthermore, it is quite difficult to separate the different enantiomers, which is an added incentive to find a method to efficiently make just one enantiomer.

As mentioned earlier, enzymes tend to use and create a specific enantiomer over the other. Therefore, a great way to ensure that one enantiomer is created over the other is to use an enzyme in creating the chiral center. For this purpose, the Masterson research

lab at the University of Southern Mississippi has been using Pig Liver Esterase (PLE) to create chiral centers (Smith et al, 2012). PLE has been found to work with an active site divided into four separate pockets envisioned as the Jones Model (Jones et al, 1990). The diester moiety fits into the front polar (P_F) and the back polar (P_B) pockets. The R group and the methyl group could fit into either the long hydrophobic pocket (H_L) or the short hydrophobic pocket (H_S). The hydrolysis occurs in the P_B pocket taken from Jones et al.'s paper (1990.) Figure 2 demonstrates Jones et al.'s findings, with the figure taken from Banerjee et al.'s paper (2012). Reaction Scheme 2 visualizes the chemical change of the diester substrate after PLE hydrolysis.

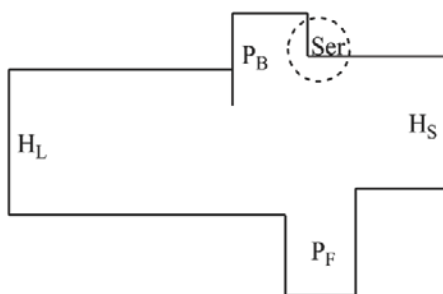
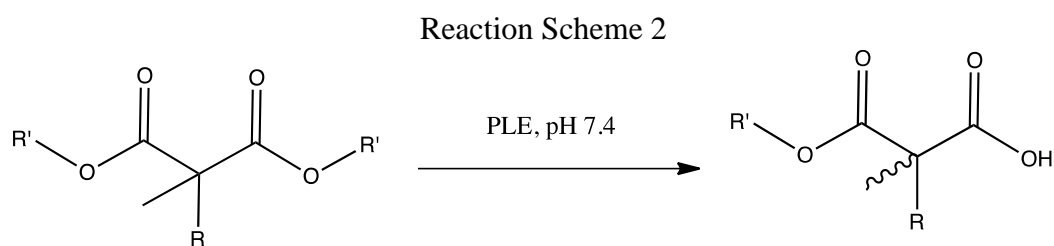


Figure 2: Jones Model of PLE Active Site



The reaction above is the general reaction scheme used in our lab for creating the chiral center. The R' group is usually either a methyl or an ethyl group. The solution is kept at a pH of 7.4, and there is a co-solvent in the solution that is usually about 2.0% ethanol. PLE hydrolysis usually uses crude PLE with all six isozymes. With diester

substrates, this hydrolysis is asymmetric, in which only one of the esters is converted into a carboxylic acid.

Isozymes are enzymes that catalyze the same reaction but have different compositions due to being encoded for by different genes (Cox and Nelson, 2008). It is important to note that before the PLE hydrolysis the compound is a diester, which is a compound with two esters. Therefore, the carbon did not have four different groups attached to it. It only had three. After PLE hydrolysis, a chiral center was created when one of the esters was converted into a carboxylic acid.

According to Smith et al. (2012), PLE becomes more much more efficient when a co-solvent such as ethanol is added to the reaction mixture. This is interesting in that the enzyme's efficiency *increased* when outside of its natural conditions. When an enzyme is taken out of its biological setting, interesting new properties can arise as seen in Smith et al.'s paper. With the addition of too much co-solvent, though, the enzyme would become too denatured to work, so there is a fine balance to the amount of co-solvent added to the solution. A co-solvent is simply the lesser solvent of a solution in which both the solvent and co-solvent act as the reaction medium.

Hydrogen Bonding with PLE Hydrolysis

Hydrogen bonding is a strong type of a dipole-dipole interaction, which has been shown to stabilize many large molecules such as proteins (Chapman, Schultz, and Thorson, 1995). In Smith et al.'s paper (2012), it is shown that the co-solvents that aided the most in creating enantiomerically enriched product, such as ethanol or isopropyl alcohol, had the capability of hydrogen bonding. Even in Niwayama et al.'s paper (1994), acetone was used as a co-solvent with their PLE mediated hydrolysis to create products

such as (-) – aristeromycin. They found that adding a co-solvent not only can improve enantiomeric excess of the PLE hydrolysis, but that a co-solvent such as ethanol can even switch the enantiopreference of some of PLE's isozymes from *S* to *R*.

Our central hypothesis for this research is that hydrogen bonding between the co-solvent and the substrate is what is responsible for the improvement in the enantiomeric excess of the PLE hydrolysis. (Enantiomeric excess is when one enantiomer is more prevalent than the other enantiomer.) Therefore, a furan ring bound to the diester in the third position has been chosen to be *R* from the PLE hydrolysis reaction above. A furan ring was chosen for three primary reasons. The first reason is that furan rings can participate in hydrogen bonding as a hydrogen bonding acceptor. The second reason is that they are fairly stable because they are aromatic rings, which are known for being stable. It should be noted that while furans are stable enough for our purposes, furan rings are among the more unstable aromatic rings known (Wright, 2001). The third reason is that the furan ring can undergo a Diels-Alder reaction, which could be used later to cyclize a compound or to connect two compounds together (Gandini, 2013).

It should be noted here that Smith et al.'s research in 2012 showed a PLE hydrolysis was done on a substrate with a phthalimide group and on a substrate with a benzyl group. Their hydrolysis involving the phthalimide group showed a much higher enantiomeric excess than the hydrolysis involving the benzyl group. Both are aromatic groups, but phthalimide had two hydrogen bond acceptors while benzene had none at all. Furthermore, it has been shown in Vasu Srevatsan's research in the Douglas Masterson research group in 2015 that the furan ring in the second position, instead of in the third

position, in the diester of Reaction Scheme 3 gives a relatively high enantiomeric excess in the PLE hydrolysis.

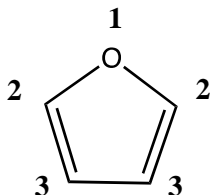
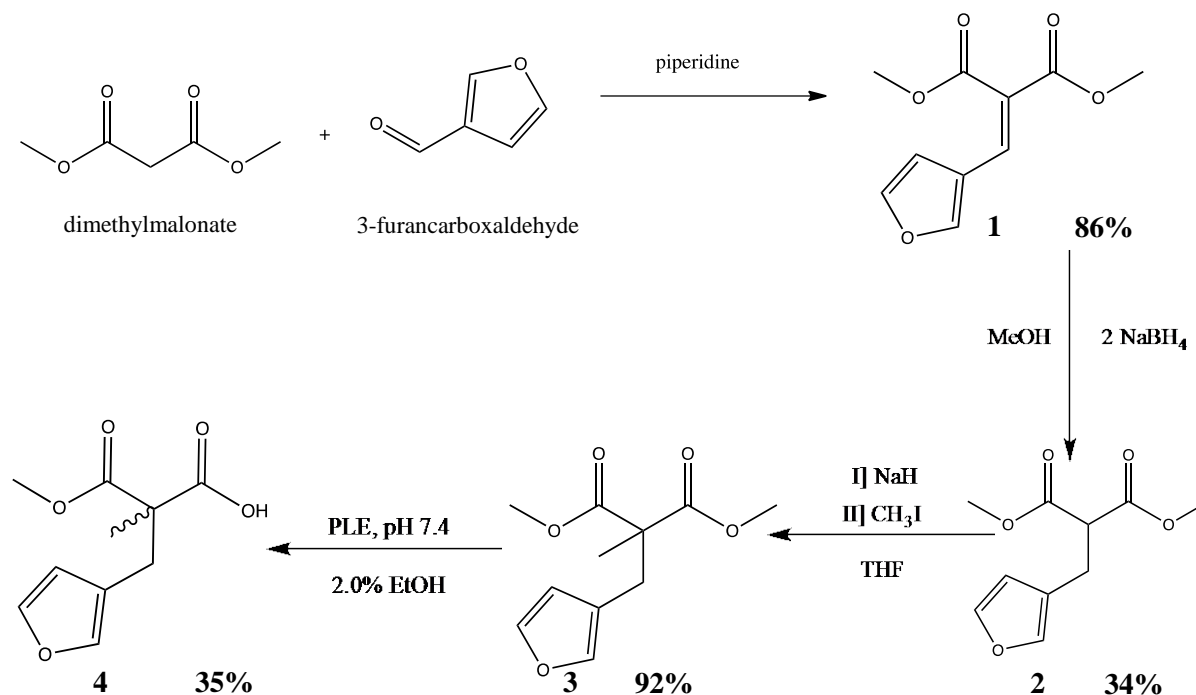


Figure 3: Furan Ring

This research desires to primarily discover if the enantiomeric excess of the PLE hydrolysis of a diester with an attached furan ring would lead to a higher than average enantiomeric excess of a diester substrate undergoing the same reaction whose R group cannot hydrogen bond. It is further desired to see if the position of the furan ring affects the enantiomeric excess of the PLE hydrolysis. This research desires to see if the enantiomeric excess of the PLE hydrolysis would significantly change if the R group could only act as a hydrogen bond donor instead of as a hydrogen bond acceptor, which will be done by making a diester analogue with a pyrrole moiety instead of a furan moiety. The research also hopes to turn the PLE hydrolysis product into an amino acid in order to investigate the properties of said amino acid in the future.

Methods

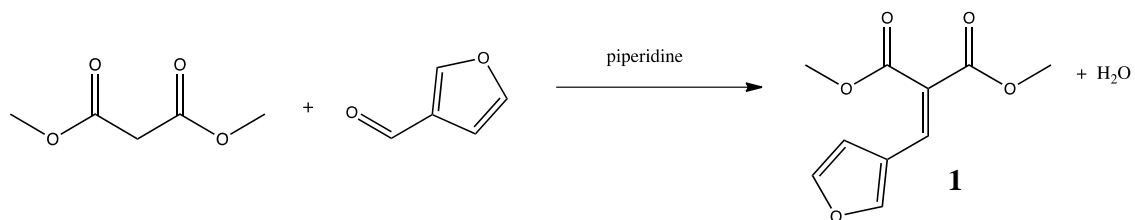
Reaction Scheme 3



Knövenagel Condensation

The first step for this research was to *make* the substrate that we need to test our hypothesis. The first reaction in the overall reaction scheme was the Knövenagel condensation. This condensation required an aldehyde (the furan), a compound with two fairly acidic hydrogens (the malonate), and a basic catalyst (the piperidine). This creates a carbon-carbon double bond between the two reactants, which also produces water as a side product.

Reaction Scheme 4

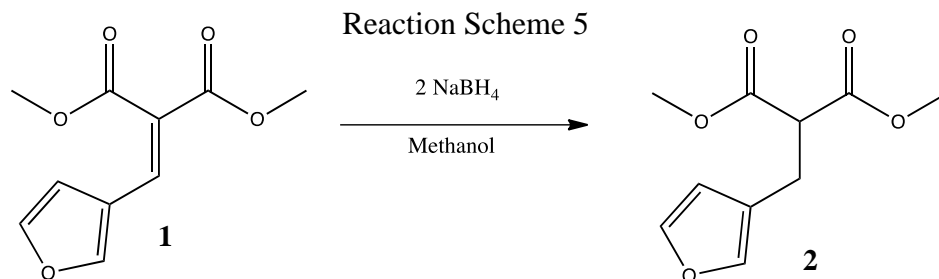


This reaction is based on the research of Edwards et al. (2002). While this specific reaction was not done by them, they ran several reactions with reactants similar to the one used in Reaction Scheme 1. They were attempting to run this condensation under solventless conditions, and they compared the results to the same reactions with a polar solvent and a nonpolar solvent. When they said “solventless,” though, they only meant that they did not *add* any material into the solution to explicitly act as a solvent. Therefore, the reactant that was most numerous was by default the solvent. However, by not adding any explicit solvent, they save money and prevent excessive waste of chemicals. In all of the reactions shown in their paper, the percent yield was greater under solventless conditions than under both the polar and the nonpolar solvent conditions. Therefore, their method for this first reaction was chosen (Edwards et al, 2002).

Sodium Borohydride Reduction

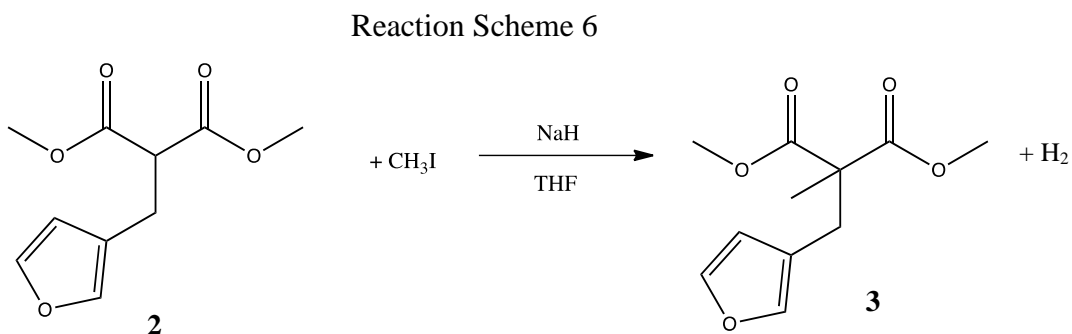
The next reaction performed was a sodium borohydride (NaBH₄) reduction to turn the double bond between the α and β carbons into a single bond according to a procedure from Bobal and Bobalova (2013). One mole of NaBH₄ can reduce 4 moles of the other reactant, so the molar ratio could have theoretically been 1:4. However, some NaBH₄ will react with the water in the air, and some of it will react to deprotonate the methanol. Therefore, two moles of NaBH₄ were used for every mole of reactant in order to keep the

NaBH₄ from accidentally becoming the limiting reagent. The solvent used was methanol in order to avoid any noticeable reactions with the ester.



Methylation of the α -Carbon

The next reaction added on a methyl group on the α carbon of the diester, and it was generally based on information learned from introductory organic chemistry (Fryhle and Solomons, 2011). This was to help ensure external validity. The purpose of this research is to see if hydrogen bonding helps to increase the enantiomeric excess of PLE hydrolysis. In the Masterson lab while doing other PLE hydrolyses, the substrates had a methyl group on the α carbon. In order to compare the enantiomeric excess of this coming reaction, the substrate should *only* differ in hydrogen bonding ability.

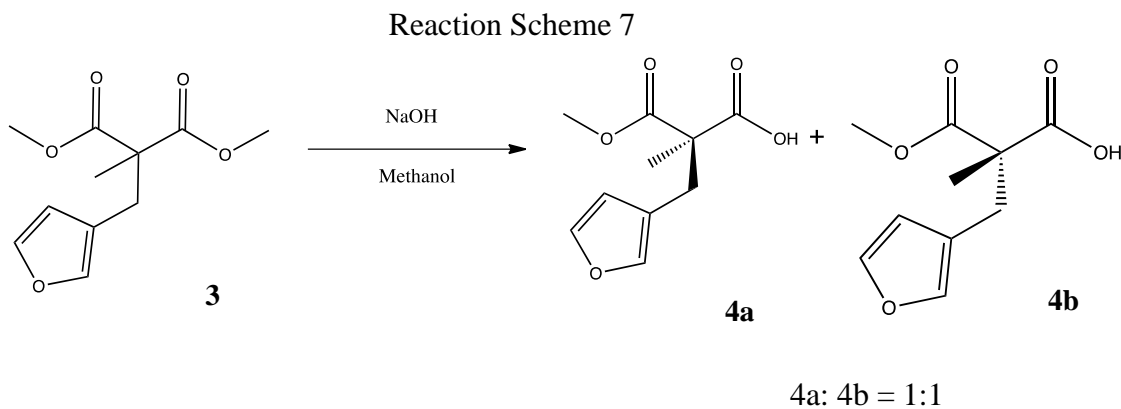


This reaction was somewhat delicate due to the sodium hydride (NaH) used. This is because NaH can also react with water vapor in order to form hydrogen gas and sodium hydroxide. Dry THF was used as a solvent to prevent unnecessary exposure to

water. Nevertheless, some of the NaH was used up while transporting it from the analytical balance to the three-necked round bottom flask. Therefore, while theoretically a 1:1 molar ratio could have been used, some excess NaH was used to compensate for the humidity. The iodomethane (CH₃I) was used in a 1:1 molar ratio, though.

Asymmetric Ester Hydrolysis

The intended substrate was then ready for PLE hydrolysis. Before it went through PLE hydrolysis, some of the substrate went through a simple hydrolysis of an exact 1:1 molar ratio of substrate and sodium hydroxide (NaOH). It then underwent the purification and analytical steps used above along with chiral high-performance liquid chromatography (HPLC) using an OJ-H column. This technique separated the enantiomers formed from this reaction in order to calculate the enantiomeric excess of just a standard hydrolysis without an enzyme like PLE.



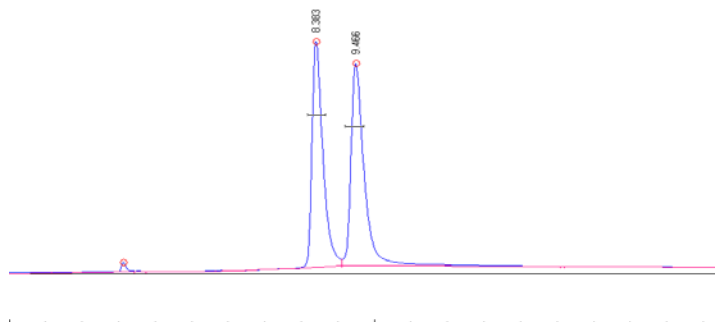
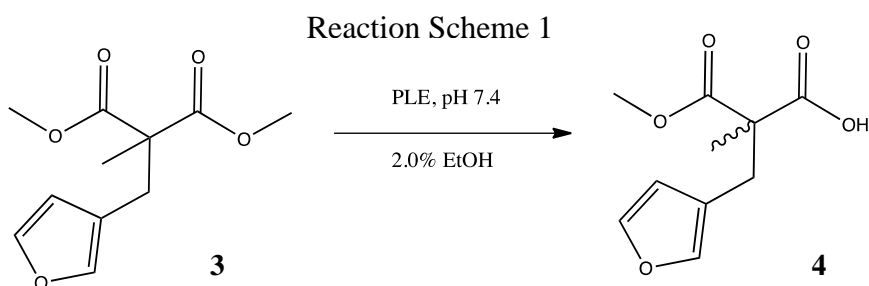


Figure 4: Chiral HPLC Chromatogram of the Racemic Mixture of Reaction Scheme 8

The PLE hydrolysis meanwhile used crude PLE instead of NaOH to perform the hydrolysis. This hydrolysis also used a small amount of co-solvent such as methanol or ethanol. The pH was maintained at 7.4 by phosphate buffer in order for PLE to work. Since the product was an acid, 1 M NaOH solution had to be titrated into the solution throughout the hydrolysis to maintain the pH. If the solution were to get below pH 4, the enzyme would be irreversibly denatured (Barker and Jencks, 1969).



This reaction was replicated numerous times, and the enantiomeric excess was analyzed each time using HPLC. Its enantiomeric excess was then compared to the enantiomeric excess of the PLE hydrolysis of Srevatsan's diester. A pyrrole analogue of Srevatsan's diester is currently being prepared using a similar method as in Reaction Scheme 3.

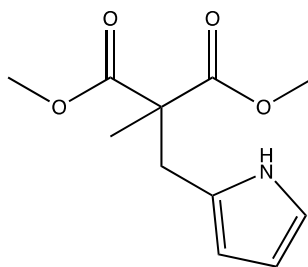


Figure 5: Pyrrole Analogue

For further reactions, the product of the PLE hydrolysis, which has a diene, can be converted into an unnatural amino acid through a variety of synthetic steps. After that, an amino acid such as glycine can be converted into a dienophile through a few synthetic steps. A dienophile is a compound that likes to react with dienes (Gandini, 2013). The dienophile could then undergo a Diels-Alder reaction to link the two amino acids together. This will be simply to see if the product of the PLE hydrolysis could be converted into an amino acid that could be used in a Diels-Alder reaction with another amino acid which would bind together the two amino acids. If that reaction is successful, it could lead to future researchers using that reaction to cyclize a peptide or to link proteins together.

Experimental Section

General Experimental Methods

Each reaction was purified using liquid-liquid extraction, in which the organic phase was washed with water and brine multiple times. After the organic phase was isolated, the solution was dried with magnesium sulfate. The solution was later filtered into a flask of known mass with gravity filtration to separate out the dry organic layer from the magnesium sulfate. The solution was then evaporated under reduced pressure to isolate pure product, and the flask with the product was then weighed in order to find out

the percent yield of the reaction. Then, the product was analyzed by a proton nuclear magnetic resonance spectroscopy ($^1\text{H-NMR}$.) This was to see if the product was made and if there were still any contaminants in the product by identifying unique protons. If the relative number of protons and their respective chemical shifts matched with the predicted values, then we knew that the product was successfully made with purity. In other words, if the height of the peaks and the position of the peaks are where they should be, then it would have gone well.

Knövenagel Condensation

With slight modifications made by Dr. Masterson and following the baseline instructions from Edwards et al.'s paper, the first step added the two reactants on a 1:1 molar ratio into a sturdy vial (2002). Then, a spin vane was added to keep the reactants mixing well once the stir plate is activated. The vial was then placed into a metal block and placed that block on top of the stir plate inside of the fume hood. Then, we added in about four drops of piperidine (a catalytic amount) into the reactant solution. This small amount of piperidine was needed because it was the catalyst of the reaction, which means it reformed at the end of each reaction. A catalytic amount was only added to save resources and also to make the purification of the product later on easier. The vial was then capped, and the stir plate was turned on to start stirring the solution (Edwards et al, 2002).

The stir plate also heated the solution to around $65\text{ }^\circ\text{C}$ for about one hour, but it was left overnight if necessary. Since the gas inside of the vial tends to expand when heated, the vial has the potential to break up due to the increased pressure. Therefore, a

sturdy vial was used, and the screen of the fume hood was closed once the reaction is started to prevent any potential injuries.

When at least one hour had passed, the heat was taken off so that the vial can cool down. After waiting about five minutes, the vial had cooled enough to be taken out of metal block and onto a cloth that can let the vial cool down further. Once that had been done, the vial was opened and liquid-liquid extraction was performed to separate the product from the piperidine and the water by-product. If there were no product sticking to the side of the vial, diethyl ether was used to help get the solution out of the vial and into the separatory funnel. If there were product sticking to the side of the vial, dichloromethane was used instead. The following workup followed the general experimental methods listed above.

Sodium Borohydride Reduction

About 9.5 grams of **1** was dissolved in about 22 mL of methanol under a nitrogen atmosphere at room temperature. The solution was stirred in a three-necked round bottom flask with a spin vane and stir plate for about four hours in an ice bath. While stirring, about five portions of NaBH_4 were added into the flask, each of about 0.86 grams. After the first portion, each of the four remaining portions were added once the solution stopped bubbling from the previous portion. Water was then added to neutralize any excess NaBH_4 (Bobal and Bobalova, 2013). Diethyl ether was the organic solvent used in the liquid-liquid extraction, and it followed the general experimental methods from there. However, the product was seen to be impure, so column chromatography was used using a solvent system of 80% hexanes and 20% diethyl ether to further purify the product for the next step.

Methylation of the α -Carbon

This reaction required a completely dry three-necked flask and spin vane. It also used a water condenser to help prevent any evaporation of the solution during the heating process. The flask was held over the stir plate in an ice bath. On top of the water condenser, there was a gas inlet to allow for the creation of an inert nitrogen atmosphere to limit any side reactions the NaH could do with the air. Then, about 0.15 g of NaH was measured and quickly placed into the flask. Because the NaH came in mineral oil, pentane was added to get the oil off of the NaH. Once the pentane had been taken off the oil, the flask needed to be tilted in order to siphon off the pentane to be disposed of.

After that, 10 mL of dry THF were added in via syringe. Then, 0.5 g of **2** was dripped in via syringe in order to avoid the solution getting too hot. Once all of the solution had been safely dripped into the flask, the reaction was monitored for about thirty minutes. Then, about 0.34 g of CH₃I was added via syringe drop by drop. The flask was then heated up to about 40 °C for about more thirty minutes. Finally, the reaction was terminated after being quenched by water. The workup for this reaction followed the general experimental methods. The product was novel, so it also had to be characterized by ¹³C-NMR, ¹H-NMR, IR (infrared spectroscopy), and HRMS (high-resolution mass spectroscopy) before advancing the project.

Asymmetric Ester Hydrolysis

The NaOH hydrolysis was performed in a small vial using a mixture of 100 mg of **3**, 0.035 mL of 50% NaOH, and 4 mL of methanol. A spin vane and stir plate were used to stir the solution. The reaction was monitored using TLC. After an hour, the reaction was finished and neutralized by an equivalent molar amount of hydrochloric acid (HCl).

The PLE hydrolysis was performed in a small beaker in phosphate buffer using a titrator that titrated in NaOH into the solution to keep the pH around 7.4. About 150 mg of **3** was added into beaker along with 1.5 mL of ethanol and 68.5 mL of phosphate buffer. About 10 mg of crude PLE were weighed and were mixed with a few drops of 3 M ammonium sulfate. When needed, a small amount of phosphate buffer was used to aid in the transfer of the PLE from the weigh boat to the reaction beaker. The reaction was determined to be complete when the titrator no longer added in NaOH. The product mixture was then filtered using vacuum filtration through a celite bed, and extracted using dichloromethane.

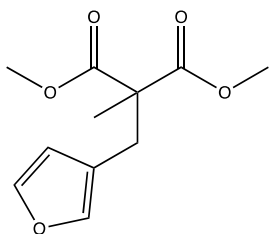
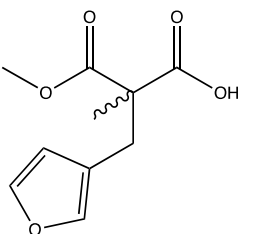
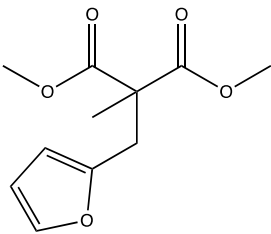
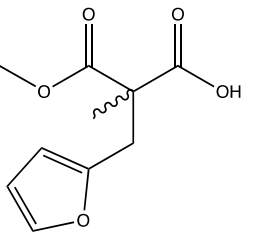
The liquid-liquid extraction for both asymmetric hydrolyses first used alkaline water (pH 8) as the aqueous phase and dichloromethane as the organic phase. This caused the product, **4**, to deprotonate and have a charge, which separated it from the unreacted diester that remained in the organic phase. Once the aqueous phase was isolated, the water was acidified to approximately pH 2. This caused **4** to reprotonate, putting it in the organic phase. Once the organic phase was washed with brine and then isolated, the general experimental methods were used for its purification and characterization. However, since both asymmetric hydrolyses created a chiral center, a chiral HPLC using an OJ-H column was needed for the product mixtures of both reactions.

Results

The diester substrate for the PLE hydrolysis, dimethyl 2-((furan-3-yl)methyl)-2-methylmalonate, was successfully made through Reaction Scheme 3. The yield for the Knövenagel condensation was around 86% while the methylation was over 90%. However, the sodium borohydride reduction gives a yield slightly above 30%, which is

due to the need to use a reductant weak enough to avoid unwanted chemistry with the furan moiety. Three different reaction conditions were used to perform the asymmetric hydrolysis on the diester substrate as can be seen in Table 1. Table 1 also contains the results of the asymmetric hydrolysis of an analogue of **3** that has the furan in the second position instead of the third, which was formed by Vasu Srevatsan during the summer of 2015 in the Masterson Research group as a part of the ACS' Project SEED in unpublished work (Srevatsan, 2015).

Table 1: Asymmetric Hydrolysis Results

Substrate (Diester I-II)	Product (Half Ester I-II)	Reaction Conditions (I-V)	% ee (HPLC)	% yield
		NaOH, MeOH	Racemic	68
		Crude PLE, pH 7.4	Racemic	37
		Crude PLE, pH 7.4, 2.0% EtOH	38	35
		NaOH, MeOH	Racemic	54
		Crude PLE, pH 7.4	67	54

As can be observed with Table 1, Substrate I's asymmetric hydrolysis yielded a racemic mixture whether NaOH or crude PLE with no co-solvent. However, Substrate

II's crude PLE hydrolysis gave a produce with 67% enantiomeric excess. Furthermore, when Substrate I underwent PLE hydrolysis with 2.0% EtOH co-solvent, the enantiomeric excess increased from racemic to 38% for Product I.

The figures below are the spectra used to confirm the data in Table 1:

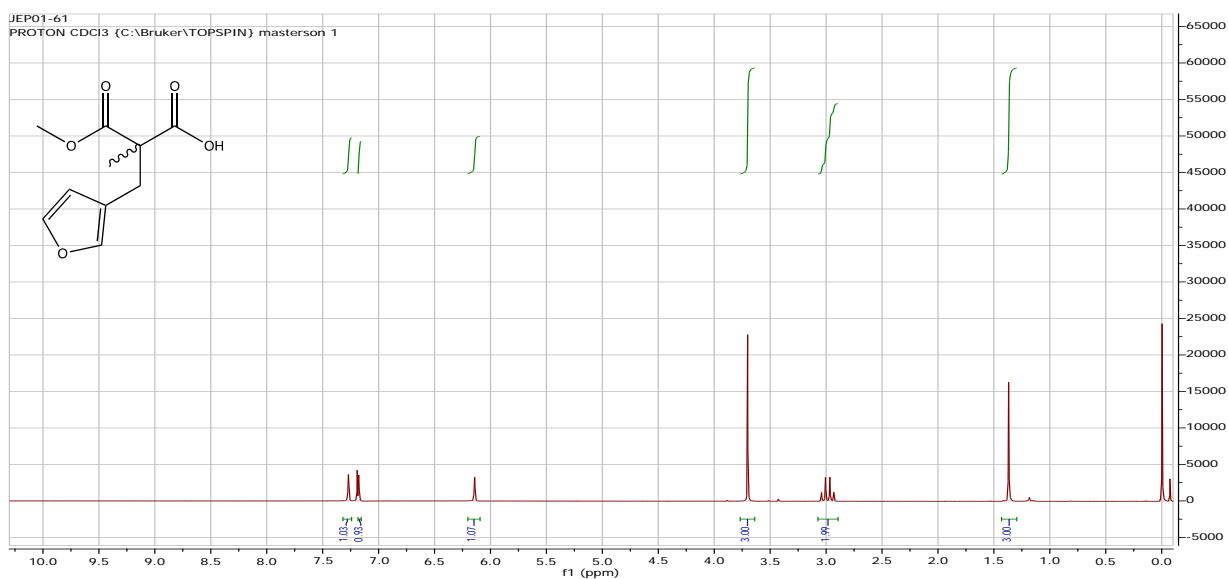


Figure 6: $^1\text{H-NMR}$ of Product I

The singlets both integrating to 1.00 around 7.3 ppm and 7.2 ppm correspond to the hydrogens of carbons attached directly to the oxygen of the furan. The other signal that merged with the singlet around 7.2 ppm is from the NMR solvent of deuterated chloroform. The singlet upfield integrating to 1.00 at around 6.1 ppm corresponds to the hydrogen at the remaining secondary carbon of the furan. The singlet integrating to 3.00 around 3.7 ppm corresponds to the three hydrogens of the methyl ester. The quartet around integrating to 2.00 around 3.00 ppm corresponds to the two hydrogens of the methylene group that is β to the half-ester. It is a quartet and not a doublet because of the chiral center causing a lack of symmetry, which is leading to this diastereotopic splitting

pattern. Finally, the singlet peak integrating to 1.00 around 1.4 ppm corresponds to the three hydrogens of methyl group at the α carbon.

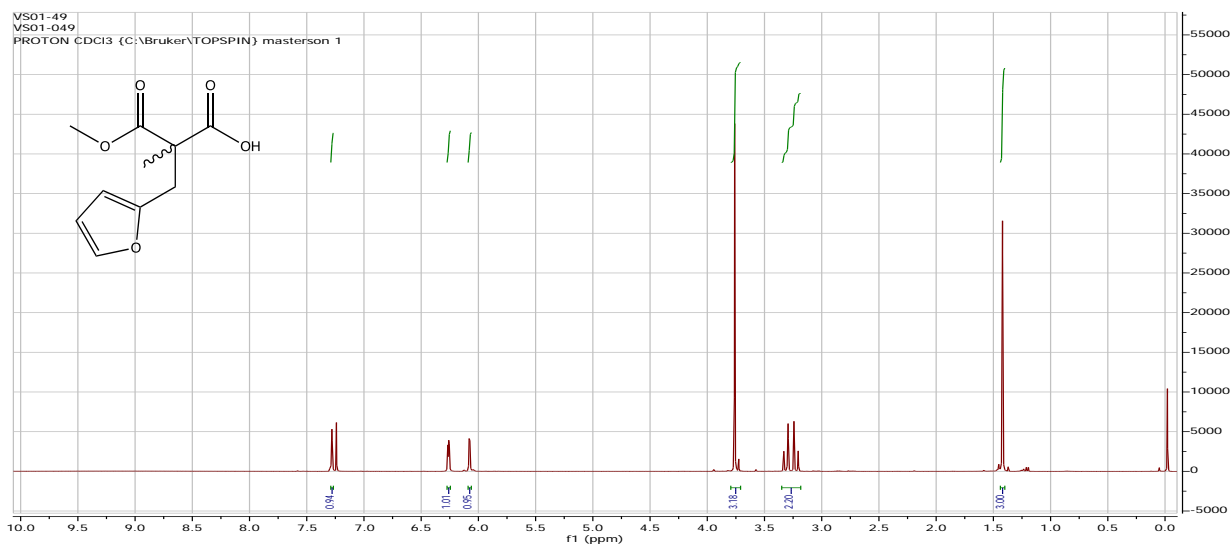


Figure 7: $^1\text{H-NMR}$ of Product II

The splitting patterns here are practically the same as they were for Figure 6. The major difference is that this analogue has a singlet further upfield around 6.3 ppm and not around 7.2 ppm. This is because there is only one carbon with a hydrogen directly beside the oxygen of the furan in Product II (Srevatsan, 2015).

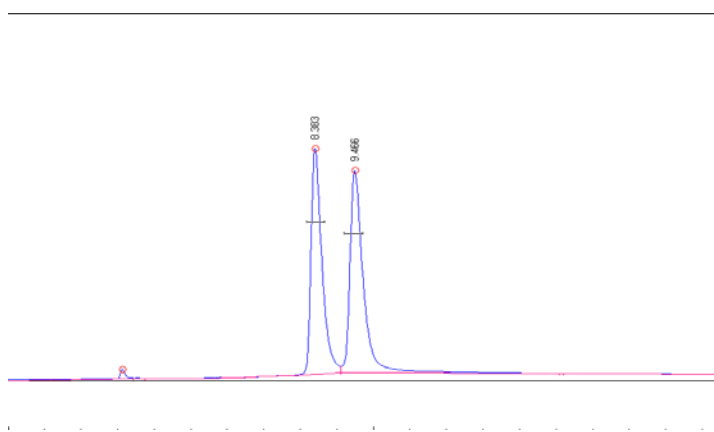


Figure 4: Chiral HPLC Chromatogram from Reaction Condition I

As can be seen from Figure 7, the two peaks integrate to give practically the same area, indicating a racemic mixture as expected from a NaOH hydrolysis of Substrate I.

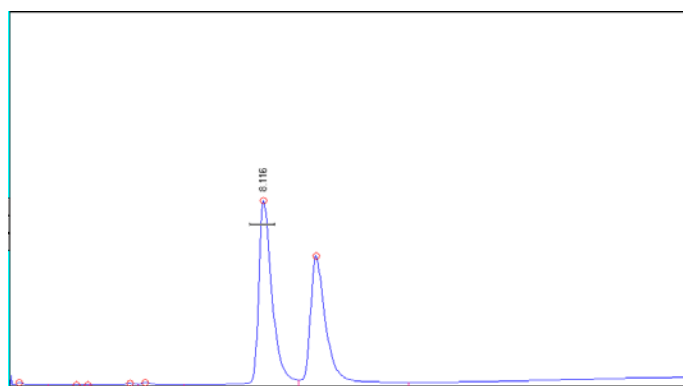


Figure 8: Chiral HPLC Chromatogram from Reaction Condition II

While the thicker base of the peak further to the left might make one assume that there was some enantiomeric excess from the co-solventless PLE hydrolysis, integration proved otherwise. Both peaks integrated to about the same, indicating a racemic mixture.

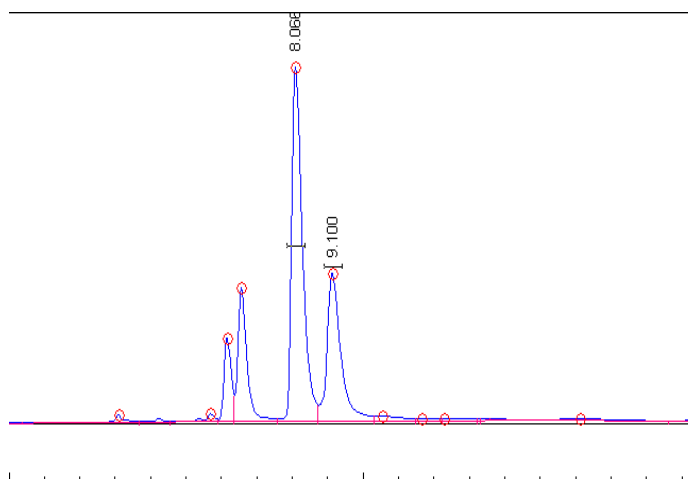


Figure 9: Chiral HPLC Chromatogram from Reaction Condition III

The peaks shown at 8.066 min and 9.100 min correspond to the relative amounts of Product I. Once integrated, they show an enantiomeric excess of around 38%. However, it should be noted that there are two other noticeable peaks to the left of the two most prominent peaks in the chromatogram. It is believed that since ethanol was used

as the co-solvent instead of methanol that some transesterification occurred during the PLE hydrolysis, where the methyl group of the half-ester product for an ethyl group. The two leftmost peaks probably correspond to the enantiomers of the half-ester product with the ethyl ester instead of the methyl ester.

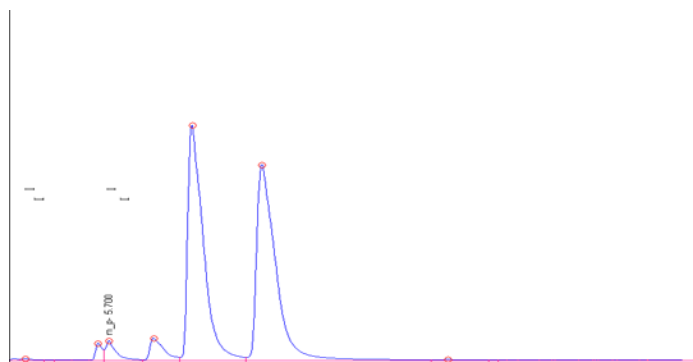


Figure 10: Chiral HPLC Chromatogram from Reaction Condition IV

As expected, Figure 10 demonstrates that NaOH hydrolysis of Substrate II resulted in a racemic mixture, as can be discovered from the integration of the two major peaks (Srevatsan, 2015).

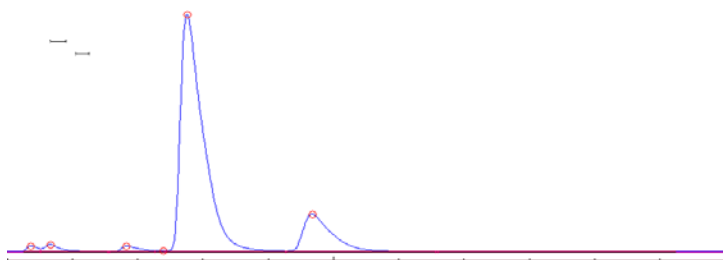


Figure 11: Chiral HPLC Chromatogram from Reaction Condition V

Figure 11 shows that there was a substantial amount of enantiomeric excess, where the leftmost major peak integrates much greater than the rightmost major peak.

Once integrated, the enantiomeric excess was determined to be around 67% (Srevatsan, 2015).

Discussion/Conclusion

It has been found that the positioning of the furan ring significantly affects the enantiomeric excess of the PLE hydrolysis. Changing the position of the furan by one carbon results can make the PLE product go from a racemic mixture to having 67% enantiomeric excess. This indicates that there may be an amino acid residue that is interacting with the R group of the diester in the long hydrophobic pocket of the PLE active site. This is further evidenced by Smith et al.'s research with the diester analogue with the pyridine moiety, which demonstrated differing enantiomeric excesses of the PLE hydrolysis depending on the three possible locations the β carbon attached to relative to the nitrogen in the pyridine ring (2015).

Furthermore, when co-solvent was added, the enantiomeric excess of the diester analogue of the furan in the third position increased from racemic to about 38%. This suggests that the co-solvent added into the reaction may be acting as a hydrogen bonding bridge between the substrate and the amino acid residue.

For future work, we are synthesizing a diester analogue with a pyrrole moiety in the second position. This will allow us to test whether or not the suspected amino acid residue can hydrogen bond with the hydrogen bond donating pyrrole as well as the hydrogen bond accepting furan. Furthermore, we will perform a Diels-Alder reaction between an appropriate dienophile and the half-ester created in Reaction Scheme 1. Our collaborator in Germany, the Dr. Uwe Bornscheuer group, is also working to model PLE

using the data this research has produced about the possible amino acid in the long hydrophobic pocket of its active site.

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